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A. M. Madl.

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The Walter and Eliza Hall Institute of Medical Research

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PROVISIONAL SPECIFICATION

for the invention entitled:

"Immunogenic Compositions and Uses Thereof"

The invention is described in the following statement:

IMMUNOGENIC COMPOSITIONS AND USES THEREOF

The present invention relates generally to an immunogenic composition comprising a glycosylphosphatidylinositol (referred to herein as "GPI") inositolglycan domain or its derivatives and more particularly to an immunogenic composition comprising a GPI inositolglycan domain from a parasite. Even more particularly, the present invention contemplates the *Plasmodium falciparum* GPI inositolglycan domain or its derivatives. The present invention is useful, *inter alia*, as a prophylactic and/or therapeutic agent in the treatment of disease conditions such as, for example, infection by parasites and in particular infection by *Plasmodium* species.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

The malaria parasite is considered to be one of the single most serious infectious agents in the world, infecting 5% of the global population and causing serious mortality and morbidity to sensitive populations and hampering socio-economic development.

Severe malaria infection shares several clinical features in common with bacterial septic shock. In both conditions, the excess production by macrophages of pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF), Interleukin-1 (IL-1) and IL-6 occurs in response to malaria "toxins" and lipopolysaccharide (LPS), respectively, leading to complications such as fever and hyperpyrexia, leukopenia, thrombocytopenia, hypotension, disseminated intravascular coagulation, leukocyte infiltration, vascular permeability and multi-organ inflammation, which may lead eventually to death. Thus,

many signs, symptons and syndromes in acute and severe malaria infection result from the activity of a parasite "toxin" released into the circulation during the blood-stage developmental cycle of the infection.

GPI has been identified as a candidate toxin of parasite origin (1-6). The structure of the molecule has been elucidated (7-9) and it comprises a lipidic domain and a glycan domain. Intact GPI occurs in two closely related forms, Pfglα (NH-CH_2-CH_2-PO_4-(Manα1-2)-6Manα1-2Manα1-6Manα1-4GlcN-H_2α1-6-*myo*-Inositol-1-PO_4-glycerol), and Pfglβ (NH-CH_2- CH_2-PO_4-6Manα1-2Manα1-6Manα1,4GlcN-H_2 α 1-6-*myo*-Inositol-1-PO_4-glycerol).

The parasite derived GPI molecule regulates host cell function and gene expression in various tissues by activating endogenous GPI-based signal transduction pathways, involving hydrolysis into second messengers and the activation of both tyrosine and serine/threonine kinases. This leads to the activation of the NFκB/c-rel family of transcription factors, which regulate the expression of numerous pro-inflammatory loci implicated in malarial pathology, such as TNF, IL-1, iNOS and ICAM-1.

In work leading up to the present invention, the inventors investigated the use of portions of GPI to induce protective immunity against malarial pathology. The inventors have surprisingly discovered that GPI portions which exclude the lipidic domain induce protective immunity whereas portions carrying the lipidic domain do not.

Accordingly, one aspect of the present invention is directed to a composition capable of inducing an immune response against a micro-organism, said composition comprising a molecule capable of inducing an immune response against a micro-organism GPI inositolglycan domain or derivative thereof but substantially incapable of inducing an immune response to a lipidic domain on a host GPI.

The present invention is predicated on the surprising observation that mice immunised with purified, intact, free GPI mount an IgM dominated response directed predominantly to the lipidic domain of the molecule, which cross reacts with host GPI lipidic domains which are exposed at host cell surfaces. The antibodies are not protective against subsequent parasite infection. Passive transfer of these antibodies exacerbates disease severity. However, immunisation with the glycan domain of malarial GPI results in IgG antibodies interactive with the glycan domain of GPI and mice thus immunised are substantially protected against pathology induced by subsequent malaria challenge. Passive transfer of these IgG antibodies is protective against pathology. The inventors have demonstrated, therefore, that IgM antibodies to the lipidic domain and IgG antibodies to the glycan domain of the malaria GPI differ in their effects, the former promoting disease and the latter preventing it.

GPIs are ubiquitous among eukaryotes, described from *T. brucei*, *T. cruzi*, *Plasmodium*, *Leishmania*, and *Toxoplasma*, as well as yeast, insect, fish and numerous mammalian sources (for recent reviews see 10, 11). GPIs consist of a conserved core glycan (Manα1-2Manα1-6Manα1-4GlcNH_2) linked to the 6-position of the *myo*-inositol ring of phosphatidylinositol (PI). GPIs are built up on the cytoplasmic face of the endoplasmic reticulum (ER) by the sequential addition of sugar residues to PI by the action of glycosyltransferases. The maturing GPI is then translocated across the membrane to the luminal side of the ER, whence it may be exported to the cell surface, free or in covalent association with proteins. The tetrasaccharide core glycan may be further substituted with sugars, phosphates and ethanolamine groups in a species and tissue-specific manner. GPI fatty acid moieties can be either diacylglycerols, alkylacylglycerols, monoalkylglycerols or ceramides, with additional palmitoylations or myristoylations to the inositol ring. The overall picture is of a closely related family of glycolipids sharing certain core features but with a high level of variation in fatty acid composition and side-chain modifications to the conserved core glycan.

Accordingly, reference herein to "GPI inositolglycan domains" should be read as including reference to all forms of GPI inositolglycan domains and derivatives thereof. The term "GPI inositolglycan" is used interchangeably with terms such as but not limited to "inositolglycan" (IG), "inositophosphoglycan" (IPG), "phosphoinositolglycan" (PIG), "phosphooligosaccharide" (POS) and the molecules described by these terms should be understood as "GPI inositolglycan" molecules.

Preferably the molecule is a portion of GPI which comprises the inositolglycan domain but subsfantially does not contain a portion capable of inducing a host immune response to a lipidic domain.

"Derivatives" should be understood to include fragments, parts, portions, chemical equivalents, mutants, homologs and analogs. Chemical equivalents of a GPI inositolglycan domain can act as a functional analog of the GPI inositolglycan domain. For example, a chemical equivalent of the GPI inositolglycan domain includes a GPI inositolglycan domain in which the phosphoglycerol component of the inositolglycan has been modified to increase hydrophobicity. This may be achieved by replacement with truncated, partial or modified fatty acids or other hydrophobic moieties and acts to improve the immunogenicity or stability of the molecule, without generating an undesirable antibody response. Chemical equivalents may not necessarily be derived from a GPI inositolglycan domain but may share certain confirmational similarities. Alternatively, chemical equivalents may be specifically designed to mimic certain immunological and physiochemical properties of the GPI inositolglycan domain. Chemical equivalents may be chemically synthesised or may be detected following, for example, natural product screening. Chemical equivalents also include synthetic carbohydrates and peptide mimics. Homologs of GPI inositolglycan domains contemplated herein include, but are not limited to, GPI inositolglycan domains from different species including, for example, Saccharomyces.

GPI inositolglycan domains suitable for use in the present invention may be derived from any natural or synthetic source. This includes, for example, GPI inositolglycan domains derived by genetic manipulation of expression systems, and by manipulations of the GPI post-translational modifications of proteins via recombinant DNA techniques such as glycosylation inhibitors.

The term "micro-organism" should be understood in its broadest sense and includes, for example, the parasitic and fungal taxa *Plasmodium*, *Trypanosoma*, *Leishmania*, *Toxoplasma* and *Candida*. Preferably, the GPI inositolglycan domain suitable for use in the present invention is a parasite GPI inositolglycan domain and even more preferably a *Plasmodium* GPI inositolglycan domain.

Accordingly, the present invention is directed to a composition capable of inducing an immune response against a parasite GPI inositolglycan domain or derivative thereof but substantially incapable of inducing an immune response to a lipidic domain on a host GPI.

Even more preferably said parasite GPI inositolglycan domain is a *Plasmodium* GPI inositolglycan domain or derivative thereof.

Most preferably, said *Plasmodium* is *P. falciparum*.

Yet even more preferably, the present invention contemplates an immunogenic composition comprising a *P. falciparum* GPI inositolglycan domain wherein said GPI inositolglycan domain comprises the structure

ethanolamine-phosphate-(Manα1,2)-Manα1,2Manα1,6Manα1,4GlcN-*myo*-inositol phosphoglycerol.

In another most preferred embodiment the present invention contemplates an immunogenic composition comprising a *P. falciparum* GPI inositolglycan domain wherein said GPI inositolglycan domain comprises the structure

X_1 - X_2 - X_3 -X_4 - ethanolamine-phosphate-(Manα1,2)-Manα1,2Manα1,6Manα1,4GlcN-*myo*-inositol-phosphoglycerol.

wherein X_1, X_2, X_3 and X_4 are any 4 amino acids.

The GPI inositolglycan domain of the present invention may be conjugated to another molecule. Said conjugation may be performed for any one or more reasons such as, but not limited to:

(i) The GPI inositolglycan domain may be too small to be antigenic. Accordingly, conjugation to a carrier molecule, such as a protein, may be required such that said GPI inositolglycan domain, which forms part of the GPI inositolglycan domain-conjugate, acts as a hapten and immunity is induced to said GPI inositolglycan domain. The carrier protein may be selected from a range of antigenic proteins such as but not limited to recombinant proteins derived from Plasmodium gene sequences, tetanus toxoid, purified protein derivative, hepatitis B or Key Hole Limpet Haemocyanin and Diptheria toxoid.

(ii) The GPI inositolglycan domain when conjugated with specific anti-pathogen vaccine molecules (such as anti-malarial vaccine molecules) may result in the production of anti-inositolglycan domain antibodies which reverse the immune suppression that otherwise may occur in response to exposure to the native form of the vaccine molecule where said molecule is itself GPI-anchored. For example, the GPI inositolglycan domain may be coupled to a malarial recombinant protein which can act as both a carrier protein and a vaccine in its own right.

Without intending to limit this aspect of the present invention to any one theory or mode of action, primary and secondary T lymphocyte responses to some GPI-anchored surface protein antigens are inhibited by the GPI anchor. Examples of such protein antigens includes Circumsporozoite (CS) proteins of *P. falciparum* and *P. berghei* and the membrane-form of Variant Surface Glycoprotein of *T. brucei*. Since immunisation against synthetic or recombinant peptides or proteins of GPI-anchored surface molecules such as the CS protein, MSP-1, MSP-2 or MSP-4, for example, may be insufficient to allow MHC Class II anamnestic boosting when the native antigens are encountered during natural parastic challenge due to the induction of immunosuppresion, immunisation against the GPI moiety provides a means to alleviate this immunosuppresion.

(iii) The GPI inositolglycan domain may comprise only part of the target epitope. For example, peptide sequences, other carbohydrates (and any associated post-translational modifications) corresponding to C-terminal domains of native GPI-anchored proteins or GPI-anchored glycosconjugates may also form part of the target GPI inositolglycan domain epitope. Removal of any part of the epitope (by removing the portion of the C-terminal domain which forms part of the GPI inositolglycan domain epitope) may lead to reduction or loss of binding of antibodies. Said peptide sequences or carbohydrates would therefore be conjugated to said GPI inositolglycan domain. For example, some antibodies to malarial GPI, while specifically neutralising GPI function, recognise epitopes which predominantly include the inositolglycan but also include portions of the protein to which the GPIs are actually bound in nature, i.e. the adjacent C-terminal portions of GPI-anchored proteins. The presence of peptide domains can also improve the affinity of certain antibodies, for example by helping to stabilise the inositolglycan conformationally. Furthermore, such conjugation can render a relatively unimmunogenic inositolglycan domain sufficiently immunogenic. Specifically, the inclusion of a C-terminal peptide determinant,

for example, may help increase the immunogenicity of the inositolglycan by forming a composite antigen which is more immunologically foreign than inositolglycan alone.

The resulting GPI inositolglycan domain-conjugate may be administered as a preparation formulated in or with an adjuvant. The adjuvant is selected from the range of adjuvants known to induce high levels of antibody, including water in oil emulsions, oil in water emulsions, water in oil in water double emulsions, saponin, Quil A extracts and other derivatives of saponin, DEAE-dextran, dextran sulphate, aluminium salts and nonionic block co-polymers. The adjuvant may include other immunomodulators, such as muramyl-dipeptide and derivatives, cytokines, and cell wall components from species of mycobacteria or corynebacteria. The adjuvant formulation may include a combination of two or more of the adjuvants listed. These lists are not to be taken as exhaustive. The selection of adjuvant is in part dependent on the species being targeted and is based on the level and duration of the immune response required and on the lack of reactogenicity (ie tissue compatibility). The level of active component and adjuvant are chosen to achieve the desired level and duration of immune response.

Host GPIs play a significant role in the normal physiological regulation of various cellular responses in higher eukaryotes. Foreign GPIs such as GPIs of parasite origin exert pathophysiological effects, and specifically regulate host cell function, by acting as a mimic of endogenous host GPI signalling pathways. Signal transduction induced in host cells by GPI's of *P. falciparum*, *T. brucei*, and *L.mexicana*, for example, activate the macrophage lineage-specific *hck* member of the *src*-family of protein tyrosine kinases within 30 seconds of addition to cells (6). Protein tyrosine kinase (PTK) activation is required for downstream gene expression resulting in phosphorylation, cell signalling and TNF, IL-1, iNOS, ICAM-1 and VCAM expression (4-6). PTK activation maps to the inositolglycan moiety of GPI and follows binding of the core glycan to a receptor on the surface of cells (6). Parasite GPIs appear to activate similar kinases as those activated upon perturbation of endogenous GPI-

anchored proteins at the cell surface indicating that they may substitute for signalling through molecules such as GPI-anchored CD16 and CD14.

The toxic nature of foreign GPIs such as parasite GPIs can be exemplified with respect to malarial GPIs. When inoculated *in vivo*, the malarial GPI induces pyrexia and symptoms of acute malaria and causes the death of the recipient in a standard assay of TNF driven lethality (1). In addition to inducing TNF and IL-1 expression in macrophages, the GPI exerts several other TNF independent effects on host tissues that may contribute to pathological processes in malaria infections. GPI directly increases expression of E-selectin, ICAM and VCAM in vascular endothelial cells (4). GPI also induces *de novo* protein synthesis of inducable nitric oxide synthase and the production of NO in a time and dose dependent manner, from macrophages and synergises with interferon-γ in this activity (15). In the hypoxic or ischaemic model, cerebral malaria is proposed to result from a blockage of the post capillary venules of the brain by sequestered parasite infected erythrocytes binding to the adhesion molecules ICAM, VCAM and E-selectin (12). GPI can therefore be lethal *in vivo* and induce malarial symptomology encompassing both systemic inflammation and organ-specific pathology such as the cerebral syndrome.

Foreign GPIs may also induce immunosuppression. GPIs isolated from *P. falciparum* and *T. brucei*, for example, when added at low concentrations to cultures of CD4+ and CD8+ α/β TCR+ T cells block cell cycle progression and cellular proliferation, inhibiting the upregulation of IL-2 R/CD25 and CD28 expression and blocking expression of IL-2, interferon γ, and IL-4. The GPIs also inhibit the T cell proliferative response to IL-2. *In vivo*, GPI anchored surface proteins such as malaria CS protein, MSP-1, MSP-2, and the membrane form variant surface glycoprotein of *T. brucei* inhibit, via the covalently associated GPI anchor, primary and secondary T lymphocyte responses to said antigens.

While not intending to limit the present invention to any one theory or mode of action, immunisation with a GPI molecule lacking the lipid domain induces an IgG response to the inositolglycan domain which blocks subsequent parasitic GPI action. Both toxicity and immunosuppression, as described above, are significantly reduced.

A further aspect of the present invention relates to the use of the invention in relation to disease conditions. For example, the present invention is particularly useful, but in no way limited to use in treating parasitic infections therepeutically or prophylactically, such as by immunizing a mammal against a parasitic infection.

Accordingly, another aspect of the present invention contemplates a method of eliciting or inducing an immune response in a mammal said method comprising administering to said mammal an effective amount of a composition capable of inducing an immune response against a micro-organism as hereinbefore described for a time and under conditions sufficient to induce an immune response.

Preferably said mammal has a disease condition and more preferably said disease condition is malaria.

According to this preferred embodiment, another aspect of the present invention contemplates a method of eliciting or inducing an immune response in a mammal having malaria said method comprising administering to said mammal an effective amount of a composition capable of inducing an immune response against a micro-organism as hereinbefore described for a time and under conditions sufficient to induce an immune response.

Without limiting this aspect of the present invention, administration of said immunogenic composition may act to result in production of antibodies which either prevent disease manifestation as hereinbefore described or affect the parasite directly, for example, by killing the parasite via binding to its surface and inhibiting its growth,

development or the onward progression of the overall infection.

An "effective amount" means an amount necessary at least partly to attain the desired immune response, or to prevent or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The term "mammal" includes humans, primates, livestock animals (eg. horses, cattle, sheep, pigs, donkeys), laboratory test animals (eg. mice, rats, rabbits, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. kangaroos, deer, foxes). Preferably, the mammal is a human or laboratory test animal. Even more preferably, the mammal is a human.

The mammal undergoing treatment may be a human or animal in need of therapeutic or prophylactic treatment of a disease condition or a potential disease condition.

In yet another aspect the present invention relates to the use of a composition comprising a molecule capable of inducing an immune response against a micro-organism GPI inositolglycan domain or derivative thereof in the manufacture of a medicament for eliciting or inducing an immune response in a mammal.

Preferably said GPI inositolglycan domain is a parasite GPI inositolglycan domain and even more preferably a *Plasmodium* GPI inositolglycan domain.

Accordingly, another aspect the present invention relates to the use of an immunogenic composition comprising a *Plasmodium* GPI inositolglycan domain in the manufacture of a medicament for eliciting or inducing an immune response in a mammal.

Preferably said mammal has a disease condition and more preferably said disease condition is malaria.

Accordingly, another aspect of the present invention relates to a vaccine composition comprising as the active component a molecule capable of inducing an immune response against a micro-organism GPI inositolglycan domain or derivative thereof, as broadly described above, together with one or more pharmaceutically acceptable carriers and/or diluents.

Preferably, said GPI inositolglycan domain is a parasite GPI inositolglycan domain and even more preferably a *Plasmodium* GPI inositolglycan domain.

Most preferably, said *Plasmodium* is *P. falciparum*.

In a most preferred embodiment, said GPI inositolglycan domain is a *P. falciparum* GPI inositolglycan domain comprising the structure

ethanolamine-phosphate-(Manα1,2)-Manα1,2Manα1,6Manα1,4GlcN-phosphatidyl-*myo*-inositol phosphoglycerol.

In another most preferred embodiment said GPI inositolglycan domain is a *P. falciparum* GPI inositolglycan domain comprising the structure

X_1 - X_2 - X_3 - X_4 - ethanolamine-phosphate-(Manα1,2)-Manα1,2Manα1,6Manα1,4GlcN-*myo*-inositol phosphoglycerol.

wherein X_1, X_2, X_3, X_4, are any 4 amino acids.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of micro-organisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of micro-organisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 μg to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5 μg to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of expressing, for example, GPI inositolglycan domain or derivative thereof. The vector may, for example, be a viral vector and it may be administered by any suitable method including, for example transfection directly into the cells of the mammal being treated or transfection into a host cell, such as a bacterium, yeast or attenuated parasite, which is then introduced into the mammal.

Administration of the immunogenic GPI inositolglycan domain of the present invention induces antibody production and in particular IgG production. Said antibodies are involved in inhibiting, halting or delaying the onset or progression of a disease condition such as, for example, pathalogical responses to a parasitic infection. Said antibodies function, for example, by neutralising parasite induced TNF induction or by direct antiperasitic effect such as killing the parasite by binding to its surface and inhibiting its growth or development or otherwise inhibiting its onward progression. Antibodies directed to the GPI inositolglycan domain or derivatives thereof may therefore also be utilised in treating parasitic infections therapeutically or prophylactically.

Accordingly, another aspect of the present invention is directed to antibodies to immunogenic GPI inositolglycan domains.

Such antibodies may be monoclonal or polyclonal, may be of any isotyope and may be selected from naturally occuring antibodies to endogenous or exogenous GPI inositolglycan domains or may be specifically raised to GPI inositolglycan domains. Antibodies may also have been raised against antigens other than the GPI inositolglycan domain but are cross-reactive with one or more epitopes of the GPI inositolglycan domain. In the case of antibodies raised to the GPI inositolglycan domain, a GPI inositolglycan may first need to be associated with a carrier molecule as hereinbefore described.

The antibodies and/or GPI inositolglycan domains of the present invention are particularly useful as therapeutic or diagnostic agents. For example, a GPI inositolglycan domain can be used to screen for naturally occuring antibodies to GPI inositolglycan domain. These may occur, for example in some infectious and autoimmune diseases. Alternatively, specific antibodies can be used to screen for GPI inositolglycan domains. Techniques for such assays are well known in the art and include, for example, sandwich assays, ELISA, Western blot and flow cytometry. Knowledge of GPI inositolglycan domain levels may be important for diagnosis of certain diseases, such as parasitic infections, autoimmune diseases (e.g. Type 1 diabetes), degenerative diseases (e.g. Type 2 diabetes) and somatically acquired genetic defects (e.g. Paroxysmal Nocturnal Haemoglobinurea) or for monitoring certain therapeutic protocols. Said antibodies would be useful as research tools or reagents for the detection of GPI inositolglycan domains. Said antibodies would also be important for example as a means for screening for levels of GPI inositolglycan domains in cell extract or other biological fluid or purifying a GPI made by recombinant means from culture supernatant fluids. Techniques for the assays contemplated herein and known in the art and include, for example, sandwich assays and ELISA, Western blot and affinity chromatography.

Antibodies to GPI inositolglycan domain of the present invention may be monoclonal or polyclonal. Alternatively, fragments of antibodies may be used such as Fab fragments. Furthermore, the present invention extends to recombinant and synthetic antibody, to antibody hybrid and to humanised antibody. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies. The antibodies of this aspect of the present invention are particularly useful for immunotherapy and immunoprophylaxis and may also be used as a diagnostic tool for assessing, for example, parasitic infection or for monitoring the program of therapeutic regimen.

It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies or synthetic antibodies) directed to the first

mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of the GPI inositolglycan domain.

Both polyclonal and monoclonal antibodies are obtainable by immunization with the GPI inositolglycan domain and are utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of a GPI inositolglycan domain, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art.

Yet another aspect of the present invention relates to a pharmaceutical composition comprising an antibody directed to a GPI inositolglycan domain together with one or more pharmaceutically acceptable carriers or diluents as hereinbefore described.

A further aspect of the present invention relates to the use of the antibodies of the present invention in relation to disease conditions. For example, the present invention is particularly useful but in no way limited to use in treating parasitic infections, their symptoms and pathologies.

Accordingly, another aspect of the present invention relates to a method of inhibiting, halting or delaying the onset of progression of a disease condition said method comprising administering to said mammal an effective amount of an antibody has hereinbefore described.

Preferably said disease condition is a parasite infection and most preferably malaria.

In yet another aspect the present invention relates to the use of an antibody in the manufacture of a medicament for inhibiting, halting or delaying the onset or progression of a disease condition.

Preferably said disease condition is a parasite infection and most preferably malaria.

The present invention is further described by the following non-limiting Figures and/or Examples.

In the Figures:

Figure 1 is a graphical representation of the epitope specificity of anti-GPI antibodies determined by competition ELISA. Sera from mice immunized with free GPI were screened for reactivity to malarial GPI in the presence or absence of defined competitors (Phosphatidylinositol or phosphatidylserine).

Figure 2 is a graphical representation of the results of C57Bl/6 mice immunized with free GPI in IFA and sham-immunized mice (IFA alone) which were challenged with *P. berghei* ANKA and survival assessed over 15 days.

Figure 3 is a graphical representation of the epitope mapping of anti-lipid monoclonal antibodies. Monoclonal antibody 1C7 to GPI derived from mice immunized with free GPI (1,5) were screened by competition ELISA for reactivity with GPI in the presence

or absence of PI and GPI glycan competitors.

Figure 4 is a graphical representation of monoclonal antibody 1C7, to malarial GPI lipid domains, recognition of mammalian GPIs at the cell surface as determined by FACS analysis. Solid line, binding of 1C7 to macrophages; dotted line, no antibody; dashed line, binding of 1C7 following PI-PLC treatment of macrophages.

Figure 5 is a photographic representation of monoclonal 1C7, to lipid domain of the GPI, induction of rapid onset tyrosylphosphorylation in host cells.

Figure 6 is a graphical representation of monoclonal 1C7 synergizy with GPI, phorbol esters and parasite extracts in the induction of TNF output from murine C3H/HeJ macrophages.

Figure 7 is a graphical representation of monoclonal 1C7 exacerbation of the *P. berghei* ANKA cerebral malaria syndrome in C57Bl/6 mice.

Figure 8 is a graphical representation of polyclonal antisera from mice immunized with the purified *P. falciparum* GPI glycan covalently conjugated to a protein carrier inhibiting TNF output from macrophages in response to GPI or total parasite extracts. Values show absorbance at 450mM by anti-TNF ELISA (Pharmingen) and are proportional to mass TNF.

Figure 9 is a graphical representation of immunization of C57Bl/6 mice with the purified *P. falciparum* GPI glycan covalently coupled to KLH providing a significant level of protection against the cerebral malaria syndrome induced by *P. berghei* ANKA.

Figure 10 is a graphical representation of GPI being the dominant TNF-inducing toxin of *P. falciparum*. Monoclonal antibodies 1G7 and 3G6 specific for the GPI glycan

derived from OVA-TCR mice immunized with OVA-glycan inhibiting TNF output from macrophages in response to total parasite extracts.

Figure 11 is a graphical representation of monoclonal antibodies to the *P. falciparum* GPI inositolglycan, upon passive transfer, substantially protecting mice against cerebral malaria and other pathologies.

Figure 12 is a graphical representation of monoclonal antibodies to the *P. falciparum* inositolglycan, upon passive transfer, protecting mice against parasite-induced lethal toxic shock.

EXAMPLE 1

REAGENTS, ANIMALS AND PREPARATION OF PARASITES

Pronase was obtained from Boehringer Mannheim. Phosphatidylinositol-specific phospholipase C was from Calbiochem. Octyl-Sepharose, Protein-G Sepharose, *n*-octylthioglucopyranoside (*n*-otg), phenylmethylsulfonylfluoride (PMSF), *p*-tosyl-L-lysine-chloromethylketone (TLCK), N-tosyl-L-phenylalaninechloromethylketone (TPCK), *p*-chloro-mercuriphenylsulphonic acid (*p*-CMPS), aprotinin, leupeptin, pepstatin, iodoacetamide, *n*-ethyl-maleimide (NEM), and Concanavalin-A were obtained from Sigma Chemical Co. Sephadex was from Pharmacia. Biogel P4 was from Biorad. Analytical or HPLC grade, acetic acid, butanol, chloroform, diethyl ether, ethanol, methanol and water were obtained from BDH and Waters. Silica G60 TLC plates were from Merck Darmstadt. Tritiated mannose, glucosamine, myristic and palmitic acids were from Amersham.

Adult female C57BL/6 and C3H/HeJ mice were bred and maintained in the Walter and Eliza Hall Institute specific pathogen free animal facility.

The FCB-1 line of *Plasmodium falciparum* were grown *in vitro* by standard methods, and confirmed free of *Mycoplasma* contamination. For the biosynthetic labelling of parasite proteins, 3H-palmitic acid conjugated to defatted bovine serum albumin in molar ratio 1:1, 3H-glucosamine or 3H-mannose were added at a final specific activity of 10μCurie/ml, to RPMI 1640 cultures of $2x10^{10}$ parasites at the late trophozoite/early schizont stage for 2 hours (for labelling of GPI precursors) or 8 hours (for labelling of protein-bound GPI). Parasites were harvested by 0.05% Saponin lysis and centrifugation in the cold at 15,000g for 20 minutes, followed by two washes in PBS and storage at -70°C.

EXAMPLE 2

PURIFICATION OF THE 195KD MSP-1 AND 56KD MSP-2 ANTIGENS

The GPI-anchored MSP-1 and MSP-2 merozoite surface proteins were purified to homogeneity as described previously (1). Biosynthetically labelled malaria parasites at the late schizont stage were lysed in 0.05% Saponin and centrifuged at 15,000g for 20 minutes, and washed as above. The pellet was extracted in 25mM *n*-octyl-thioglucopyranoside (*n*-otg), 1% BSA, 1mM EDTA, 0.1mM EGTA, 1mM PMSF, 1mM TPCK, 0.1mM TLCK, 5mM pCMPS, 1μg/ml pepstatin, 1μg/ml leupeptin, 1mM NEM, 5mM iodoacetamide, 150mM NaCl, 25mM Tris/HCl pH 7.4 by sonication on ice. The extact was clarified by centrifugation at 20,000g for 30 minutes in the cold, and the supernatant decanted and loaded onto two immunoaffinity columns arranged in sequence, containing approximately 10mg monoclonal antibody 111.4 or monoclonal antibody 113.1, each cross-linked to Protein G-Sepharose by gluteraldehyde (all procedures on ice). The protein extract was passed through the column at a rate of 0.3ml/min. The columns were washed first with with 100ml 10mM n-otg, 1% BSA, 300mM NaCl, followed by 100ml 10mM n-otg, 300mM NaCl. Antigen was eluted from each column with four column volumes of 10mM n-otg, 200mM glycine pH 2.8. The pH of the eluate was neutralized with 2M Tris. Aliquots of protein were analysed for purity by SDS-PAGE followed by staining with Coomassie brilliant blue. The remaining purified proteins were dialysed exhaustively against 100mM NH_4HCO_3 using dialysis membrane previously boiled exhaustively in 10mM EDTA followed by boiling in 10 changes of double distilled water. Protein concentration was determined by standard methods.

The remaining detergent soluble extract was made up to 1mM $CaCl_2$, 1mM $MgCl_2$, and 1mM $MnCl_2$, and passed over a Con-A sepharose column, followed by washing with 10 column volumes of extraction buffer. The column was first eluted with detergent buffer containing 0.5M α-methyl-mannopyranoside and 0.5M α-glucopyranoside, followed by 25mM *n*-otg in 8M urea. Aliquots were subject to SDS-PAGE and

fluorography or staining with Coomassie blue.

EXAMPLE 3

PURIFICATION OF THE C-TERMINAL GPI ANCHORS OF DEFINED PARASITE ANTIGENS

To purify the intact C-terminal GPIs free of detergent, non-covalently bound lipids, glycolipids, phospholipids and protein or peptide fragments, affinity purified MSP-1 and MSP-2 were first scrubbed with organic solvents. 10mg/ml GPI-anchored proteins were placed in 150μl aliquots in clean glass tubes. 600μl MeOH was added and vortexed, followed by 150μl l Chloroform and 450μl water and further vortexing. The samples were centrifuged at 14000rpm for 3min, the supernatant discarded, and the interphase and lower phase mixed with 450μl MeOH and re- centrifuged at 14000rpm for 3min. The protein pellet was extracted 5 times with C/M/W 10:10:3, and finally extracted with acetone over night at -20°C. The acetone was removed completely and the proteins taken up with sonication in 6M Urea, 1mM DTT, 1mM iodoacetic acid. After 15 minutes at room temperature, the sample was diluted 6 fold and made to 5mM $CaCl_2$. 2.5% pre-digested Pronase B was added for 72h at 37°C with 2 additions of 0.3% pronase. The digested sample was phase separated between water and water-saturated butanol, and the organic phase back extracted with water. The butanol phase was spotted onto TLC plates (Si-60) and run in the solvent system C/M/HAc/W 25:15:4:2. The pronase-digested GPI fragment free of contaminants remains close to the origin, and was detected by Berthold Digital Autoradiograph. The appropriate region was scraped and the material eluted twice with C/M/W 10:10:3 followed by 40% 1-propanol in water. The material was dried under nitrogen gas, and once more separated between water and water-saturated butanol.

EXAMPLE 4

PURIFICATION OF GIPLs AND GPI BIOSYNTHETIC PRECURSORS BY TLC

GPI biosynthetic intermediates and non-protein bound mature GPI species were purified by TLC. $2x10^{10}$ *P. falciparum* schizonts were labelled with 1mCi ^{3}[H]-mannose or ^{3}[H]-palmitic acid in 250ml glucose deficient RPMI 1640 supplemented with 40mM fructose and 0.5% Albumax (GIBCO) for 2 hours. Parasites were harvested by saponin lysis and washed twice in PBS. They were extracted three times in chloroform/methanol (2:1) and three times in chloroform/methanol/water (1:1:0.3). The chloroform/methanol extracts were subject to repeated Folch washing and the chloroform phase dried in a Speedvac. The chloroform/methanol/water extracts were dried down and partitioned between water and water-saturated butanol. The butanol phase was washed with water and dried in a Speedvac. Both residues were then separated by TLC and the plates scanned by Bertold Digital Autoradiograph TLC scanner. The radiolabelled peaks were identified and removed by scraping and re-extraction followed by drying. Areas lying between and outside the identifiable peaks were treated in the same way, as were sham plates. In some experiments, the GPIs were further purified over Octyl-Sepharose. Samples were taken up in 5% 1-propanol in 100mM Ammonium acetate and loaded at a flow rate of 0.1ml/min onto an Octyl-Sepharose column, and the column washed with 100mM Ammonium acetate, 5% 1-propanol. The column was eluted in a gradient running from 100mM Ammonium acetate, 5% 1-propanol to 60% 1-propanol in water. GPI containing fractions were lyophilised and flash evaporated in methanol.

EXAMPLE 5

GENERATION OF CHEMICAL AND ENZYMATIC HYDROLYSIS FRAGMENTS OF GPIs

Purified, glucosamine-labelled *P falciparum* GPIs, in which all dpms were detected in the organic phase following butanol/water partitioning, were subject to base hydrolysis

by suspension in methanol/ammonia 1:1 for 6 hours at 50°C, followed by partioning between water and water saturated butanol. Essentially 100% of label was then recovered from the aqueous phase. The aqueous phase was twice extracted with water-saturated butanol, lyophilized, and flash evaporated with methanol.

EXAMPLE 6

DEAE ANION EXCHANGE CHROMATOGRAPHY

GPIs were loaded onto a A DEAE column in 99% methanol, 1% water and washed with ten column volumes of solvent. They were subsequently eluted in 100mM Ammonium Acetate in 99% methanol, 1% water and dried under Nitrogen.

EXAMPLE 7

BIOGEL P4 SIZE-EXCLUSION CHROMATOGRAPHY

Base-hydrolysed GPI glycans were spiked with phenol red and blue dextran in 100mM Ammonium Acetate and further size-fractionated by passage through a 1cm x 1.2 metre Biogel P4 column equilibrated in 100mM Ammonium acetate in water. The column had previously been exhaustively calibrated by repeated analytical runs with GPI mixed with acid hydrolysed dextran markers to yield the relative elution position of glucose units detected by staining with orcinol in concentrated sulfuric acid. The column runs proved to be highly reproducible. For preparative purposes the dextran markers were omitted. The GPI peak was detected by scintillation counting of aliquots.

EXAMPLE 8

COMPOSITIONAL ANALYSIS BY GC/MS

Glycan concentration and compositional purity was determined by GC-MS, following acid methanolysis and trimethylsilyl (TMS) derivatization. *myo*-Inositol content was measured following acid hydrolysis (6N HCl, 110oC, 16 h) and TMS derivatization,

with selected ion monitoring for *m/z* 305 and 318. *scyllo*-Inositol was used as internal standard throughout.

EXAMPLE 9

COUPLING OF GPI GLYCAN TO MALEIMIDE-ACTIVATED KLH

The GPI glycan was exposed to 1mM Traut's reagent (2-iminothiolane) in 60mM triethanolamine, 7mM potassium phosphate, 100mM NaCl, 1mM EDTA, pH 8.0 in the cold for 90 minutes under nitrogen. The sample was then desalted by gel filtration at 4°C through a small Biogel P4 column equilibrated in 7mM potassium phosphate, 100mM NaCl, 1mM EDTA, pH 7.2 and added to maleimide-activated KeyHole Limpet Haemocyanin (KLH) or Ovalbumin (OVA) in coupling buffer (7mM potassium phosphate, 100mM NaCl, 1mM EDTA, pH 7.2) overnight. The degree of conjugation was estimated by comparison of cpms before and after dialysis of the sample against PBS, or by use of Ellman's reagent for the quantitation of sulfhydryl groups. Excess reactive sites were blocked with cysteine.

EXAMPLE 10

EPITOPE MAPPING OF ANTI-GPI ANTIBODIES

Coupling of the purified GPI glycan to proteins was undertaken as above. To measure anti-lipid reactivities, we utilized commercially available phosphatidylinositol from Sigma with identical composition to the malarial GPI, namely dipalmitoyl-PI. 2mg PI was coupled to defatted BSA according to published protocols (13).

EXAMPLE 11

ELISA ASSAY

Antigen (GPI-OVA, Glycan-OVA, BSA-PI, OVA or BSA alone) at 20μg/ml in phosphate binding buffer was incubated overnight in 50μl volumes in flat-bottomed

Immunlon 96-well plates, followed by extensive washing with buffer. The plates were blocked with 1% BSA, 1% OVA in PBS for several hours. From a 1/32 dilution, sera were titrated two-fold in 1% BSA, 1% OVA in PBS, and 50μl aliquots incubated in triplicate for 2 hours at room temperature, followed by extensive washing with 1% BSA, 1% OVA 0.05% Tween-20 in PBS. An aliquot of affinity purified, biotin-labelled isotype specific goat anti-mouse second antibody was incubated as above, followed by further washing and the addition of streptavidin-alkaline phosphatase. After 30 minutes the plates were washed again and colourimetric development initiated by the addition of p-Nitrophenylphosphate in diethanolamine buffer. Background binding to BSA/OVA-coated plates was determined in parallel. The end-titres derived are the last point giving values statistically different by two-way analysis of variance from non-specific binding by the same serum to the BSA/OVA-coated plates.

EXAMPLE 12

COMPETITION ELISA

From a 1/32 dilution, sera or mAbs were titrated two-fold in 1% BSA in PBS, 0.05% Tween-20, and pre-incubated for 4 hours at room temperature with a molar excess of competitior (20μg/ml PI, or phosphatidylserine (PS), or diluent alone). Antigen (BSA-PI or BSA alone) at 20μg/ml in phosphate binding buffer was incubated overnight in 50μl volumes in flat-bottomed Immunlon 96-well plates, followed by extensive washing with buffer. The plates were blocked with 1% BSA in PBS for several hours. 50μl aliquots of titrated antibody with or without competitor were incubated in triplicate for 2 hours at room temperature, followed by extensive washing with 1% BSA 0.05% Tween-20 in PBS. An aliquot of affinity purified, biotin-labelled isotype specific goat anti-mouse second antibody was incubated as above, followed by further washing and the addition of streptavidin-alkaline phosphatase. After 30 minutes the plates were washed again and colourimetric development initiated by the addition of p-Nitrophenylphosphate in diethanolamine buffer. Background binding to BSA-coated

plates was determined in parallel. The end-titres derived are the last point giving values statistically different by two-way analysis of variance from non-specific binding by the same serum to the BSA-coated plates.

EXAMPLE 13

PRODUCTION OF MONOCLONAL ANTIBODIES

Monoclonal antibodies to the lipid domain of the GPI were produced as previously described (5). Monoclonal antibodies to the glycan were generated by immunization of OVA-TCR transgenic mice on a Balb/c background with OVA-glycan, followed by fusion and screening of hybridoma culture supernatants against BSA vs. BSA-glycan.

EXAMPLE 14

MACROPHAGE CULTURE AND TNF OUTPUT

LPS-nonresponsive C3H/HeJ macrophages were obtained as previously described (1,5). $2x10^5$ adherent cells/well were given medium alone or test agents. 3 hrs after incubation TNF-α levels in the supernatant and standard curve were determined by capture ELISA (Pharmingen).

Tyrosine phosphorylation.

Rapid onset tyrosylphosphorylation was determined as previously described (6).

EXAMPLE 15

PI-PLC TREATMENT AND FACS ANALYSIS

$2x10^5$ cells were exposed to 1U/ml PI-PLC at 37°C for 2 hours, followed by washing. They were then incubated in ice cold murine tonicity RPMI 1640 with 0.05% Sodium azide and 1% BSA with monoclonal antibodies or murine sera followed by washing and a further incubation with isotype-specific FITC-conjugated antibody to mouse

immunoglobulins. After washing in the same medium the cells were counter-stained with 0.5μg/ml propidium iodide and analysed by FACSscan.

EXAMPLE 16

IMMUNIZATION OF MICE WITH FREE GPI

Mice were immunized by three successive boosts of free intact malarial GPI emulsified in Incomplete Freund's Adjuvant spaced two weeks apart. Control mice received an equal amount of IFA alone. After immunization, sera were bled and the titres of anti-GPI antibodies determined by ELISA. All animals immunized with GPI developed broadly similar levels of anti-GPI antibodies (range 1/1024 - 1/4096) among individual animals. The anti-GPI response was predominantly IgM, and epitope mapping studies by competition ELISA revealed that the antibody response was directed predominantly towards the lipidic (phosphatidylinositol, PI) domain of the molecule, with some cross-reactivity to other phospholipid determinants (Fig. 1). Two weeks after the final boost mice were challenged with P. *berghei* ANKA. Parasitaemia, the development of neurological complications, and mortality were recorded daily. No difference in parasitaemia was observed. In the control group, 100% of animal manifested between day 5 and 9 an aggressive cerebral syndrome with neurological signs proceeding to rapid death with 12 hours. In animals immunized with intact free GPI, however, deaths occurred at a noticable faster rate (Fig.2).

The increased death rate in animals immunized with free GPI and subsequently challenged with malaria may result from unanticipated autoreactivity of anti-GPI antibodies. A panel of IgM monoclonal antibodies was derived from mice immunized with free GPIs. mAbs selected at random from this panel were shown by PI-specific ELISA to be reactive with PI domain of the molecule (Fig. 3), as was expected given the established serological specificity of the polyclonal sera of the donor immunized mice (Fig. 1). In addition, these mAbs and the polyclonal antisera of GPI-immunized mice were shown by FACS analysis to react with host GPI molecules expressed at the

cell surface. Although suprising, the recognition of GPI-associated lipidic determinants at the cells surface is not without precedence (14). Pre-treatment of host cells with phosphatidylinositol-specifc phospholipase C resulted in loss of binding of these mAbs, demonstrating formally that a lipidic moiety of GPI molecules is exposed at the cell surface and is accessible for binding by autoreactive antibodies generated in response to exposure to free malarial GPI (Fig. 4). The binding was also shown to cause massive rapid onset intracellular tyrosine phosphorylation (Fig. 5), a well-known and predictable consequence of cross-linking host GPIs at the cell surface (15, 16). Following binding of these antibodies to macrophages, the cells responded more vigorously to stimulation with GPI, phorbol esters or malaria parasite extracts (Fig. 6). This particular latter finding is understandable as we have shown that malarial GPI and parasite extracts induce macrophage activation through signaling pathways involving the activation of tyrosine kinases (6), and massive upregulation of tyrosine kinase activity may potentiate the cellular response to these agonists. Upon passive transfer into mice, these mAbs were sufficient to cause an increased rate of death as compared with control IgM mAbs (Fig. 7). Thus to summarize: (i) immunization of mice with the free *P. falciparum* GPI generates IgM reacting predominantly with the PI domain of the GPI; (ii) this immunization appears to exacerbate the *P. berghei* cerebral malaria syndrome; (iii) exarcerbated pathogenicity as detected by increased death rate was also observed upon passive transfer of IgM monoclonals with the same reactivity; (iv) the mAbs were shown to cross-react with host GPIs by FACS analysis, thereby causing massive intracellular tyrosylphosphorylation and sensitization of macrophages resulting in increased TNF output in response to addition agonists. Therefore we propose a novel mechanism by which the acquisition of certain auto-reactive immunological specificities results in increased physiological sensitization to malarial toxins. This phenomenon may occur in humans leading to enhanced susceptibility to clincal disease.

EXAMPLE 17

IMMUNIZATION OF MICE WITH THE GPI GLYCAN CONJUGATED TO KLH

Previous publications dealing with the prospect of anti-disease vaccines against malaria have proposed immunizing against a phospholipid domain within the putative toxin (17, 18, 19, 13, 20, 21, 22). Our data indicate strongly that this may be deleterious and should be avoided. We sought therefore to develop a novel approach, namely to detoxify and deacylate the GPI and to determine whether immunization against the glycan domain of the molecule would exarcerbate disease or be sufficient to protect mice against malarial pathology. Mice (n=7) were immunized by three successive boosts of 50μg KLH-glycan emulsified in Incomplete Freund's Adjuvant spaced two weeks apart. Two separate control groups (n=8 each) comprised animals receiving an equal amount of sham conjugated KLH in IFA, or those left untreated. After immunization, sera were bled and the titres of anti-GPI antibodies determined by ELISA. All animals immunized with KLH-glycan developed detectable anti-GPI glycan IgG antibodies, although there were differences in end-titre (range 1/128 - 1/4096) among individual animals. The sera from vaccine recipients (but not sham-KLH controls) were able to inhibit TNF output from macrophages stimulated with crude *P. falciparum* extracts, providing convincing proof-of-principle for the neutralization of pathogenicity (Fig. 8). In contrast to the host-reactive antibodies to the GPI lipid domain, pre-exposure of macrophages to these sera did not result in increased TNF output in response to additional agonists. With these sera we were unable to detect significant cross-reactivity with host GPIs at the cell surace as judged by FACS analysis of antibody binding to host cells.

The *P. berghei* ANKA murine cerebral malaria model has many features in common with the human cerebral malaria syndrome. It is a TNF-α and interferon-γ(IFN-γ) dependent encephalitis associated with upregulation of ICAM-1 on the cerebral microvascular endothelium, an increase in both parasite and macrophage/neutrophil

adherence to these target cells, and attendant neurological complications. Unlike human cerebral malaria, there is a breakdown of the blood-brain barrier in the terminal stages of the murine syndrome. However, in the proximal stages the murine disease reflects more accurately the inflammatory cascade leading to cerebral involvement in humans. To determine whether anti-GPI immunization prevents cerebral pathogenesis *in vivo*, mice were immunized with *P. falciparum* IPG conjugated to KLH. Two weeks after the final boost mice were challenged with *P. berghei* ANKA. Parasitaemia, the development of neurological complications, and mortality were recorded daily. No difference in parasitaemia was observed among groups. In both control groups, 87.5% of animal manifested between day 7 and 12 an aggressive cerebral syndrome with neurological signs proceeding to rapid death with 12 hours, and 12.5% did not develop the syndrome. As there were no significant differences between sham-immunized and untreated groups, the data from these two control groups are pooled (Fig.9). In recipents of KLH-glycan, one animal (14.2%) died with similar kinetics, two animals (28.5%) developed the cerebral syndrome with sustantially delayed kinetics (on days 10 and 11, and showing prolonged course of syndrome before succumbing), and four animals (57.2%) were completely protected, failing to develop the cerebral syndrome at any stage (Fig.9). Thus immunization of mice with the *P. falciparum* GPI glycan covalently linked to a carrier protein affords substantial protection against the *P. berghei* cerebral malaria syndrome.

A panel of monoclonal antibodies was made from mice immunized with purifed GPI glycan conjugated to OVA (OVA-glycan). The hybridoma fusion products were initially screened for binding to BSA-glycan as compared to BSA alone. Over 80 glycan-reactive IgG monoclonal antibodies were detected. Of these, many were reactive with parasites but not host erythrocytes as judged by the Indirect Fluorescent Antibody Test. Purified monoclonal antibodies 1G7 and 3G6 were sufficient to block the induction of TNF by 100% when added at low concentration to total crude parasite extracts (Fig. 10). To determine whether anti-GPI antibodies alone are sufficient to prevent severe malarial pathology, mice were infected with 10^6 *P. berghei* ANKA i.p.

On day 4 they were divided at random into 10 controls receiving an irrelevant IgG and groups of 5 receiving mAbs 1D12, 2C4, 3G5 and 4C3 raised against the *P. falciparum* GPI inositolphosphoglycan. All mice received 100µg antibody/day i.p. for 7 days. Mice were monitored for parasitaemia daily and clinical signs every 6 hours. 100% of controls died of the cerebral malaria syndrome between days 6 and 8 post-infection. Throughout this period, no animals receiving either of the 4 anti-GPI monoclonal antibodies showed signs of illness, despite being equally parasitized as controls. On day 10 one of the 5 animals receiving monoclonal 3G5 died. Other than this individual, no others showed cerebral signs and none died (Fig. 11). Thus 19/20 (95%) of the 20 animals receiving anti-GPI mAbs survived, vs. zero survival in controls (n=30 total). Parasitaemias were identical in test and control groups throughout the experiment. For visual clarity, the figure shows the 4 treatment groups in aggregate. In addition, 5 mice received antibodies alone without parasite challenge. There were no detectable acute or toxic reactions in these mice receiving antibodies alone.

In addition a standard murine model of TNF-driven lethality was used to determine whether GPI mediates parasite-induced acute toxic shock. This model manifests disseminated intravascular coagulation, peripheral vascular failure and shock, and thus has clinical features in common with the human "algid malaria" syndrome. LPS-non-responsive C3H/HeJ and C57B16 mice were primed with 20 mg D-galactosamine followed after 1 hour by purified GPI, PE or PBS alone. Mice receiving D-galactosamine followed by vehicle alone showed 100% survival. Both PE and purified GPI induced lethal shock in 100% of D-galactosamine-primed C3H/HeJ and C57B16 recipients. mAbs to the GPI glycan substantially prevented TNF-driven lethality *in vivo* (Fig. 12).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The

invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more said steps or features.

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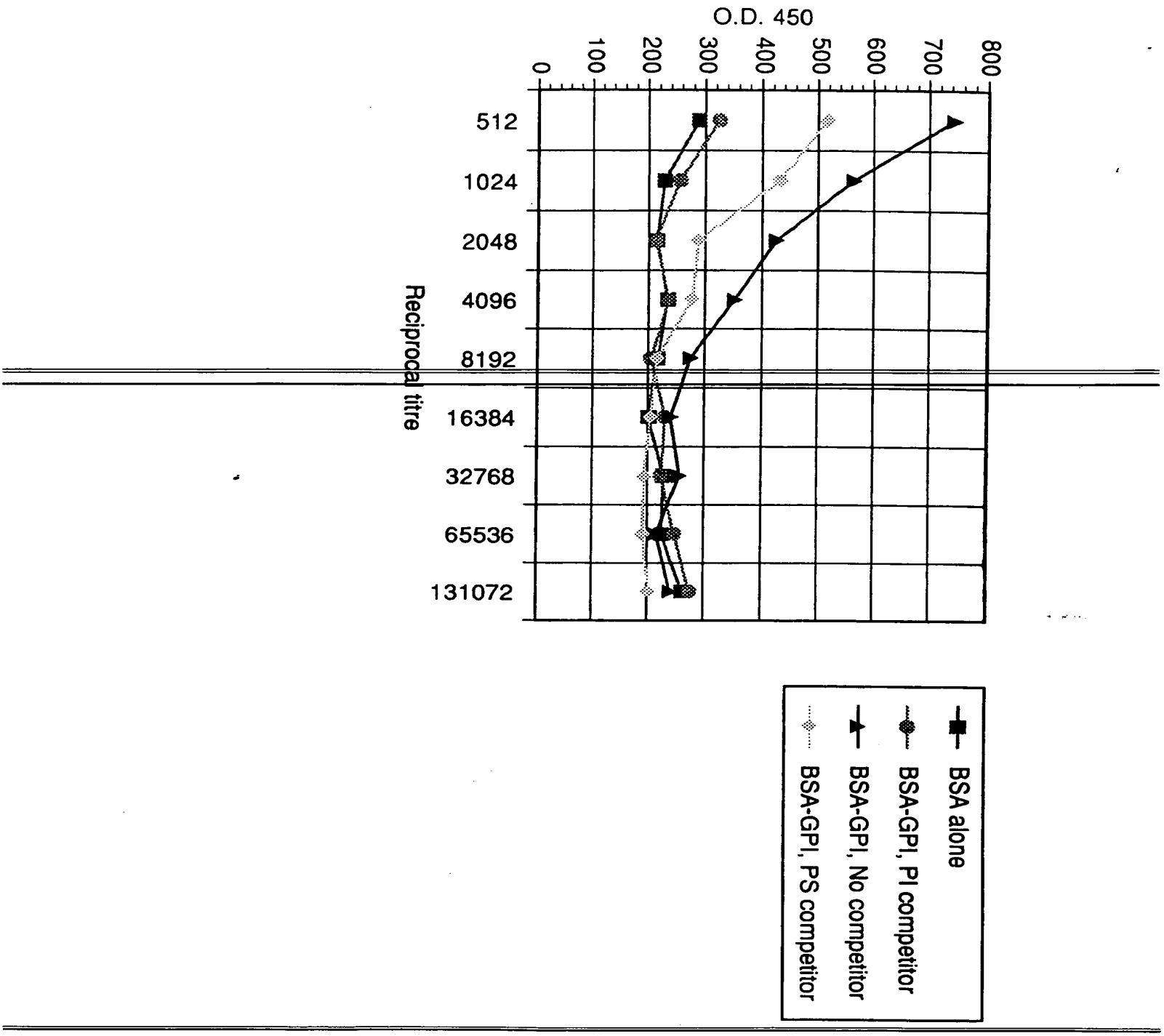
The Walter and Eliza Hall Institute of Medical Research
by their Patent Attorneys
DAVIES COLLISON CAVE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100
200
300
400
500
600
700
800
Reciprocal titre
512
1024
2048
4096
8192
16384
32768
65536
131072
BSA alone
BSA-GPI, PI competitor
BSA-GPI, No competitor
BSA-GPI, PS competitor

FIGURE 1

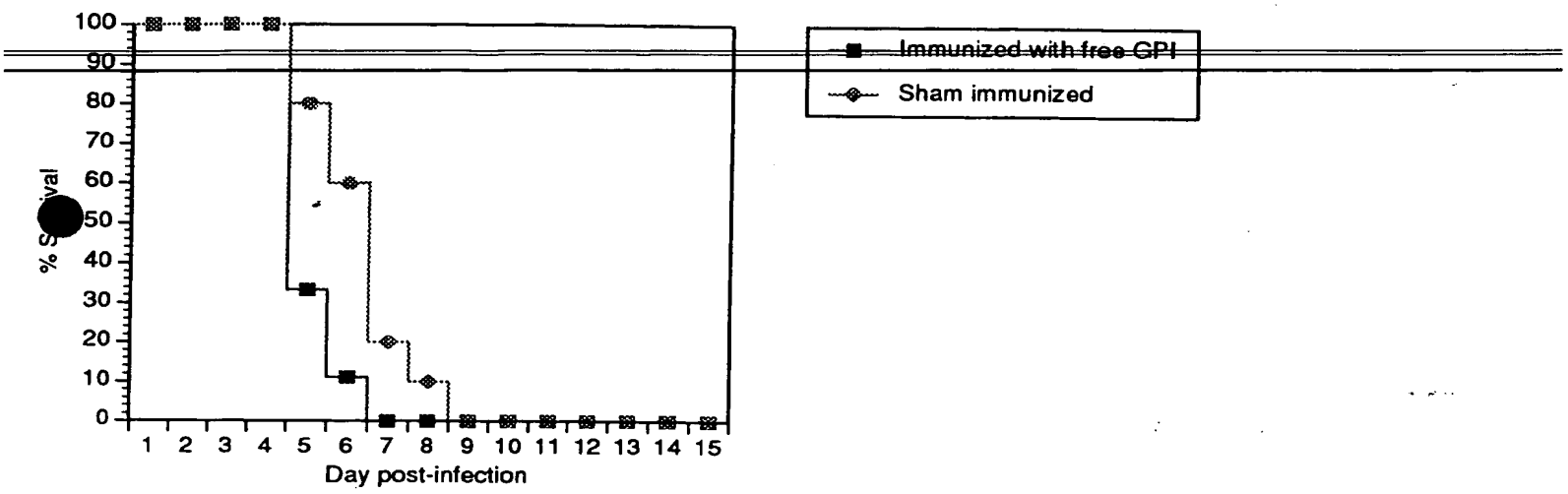
100
90
80
70
60
50
40
30
20
10
0
% Survival
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day post-infection
Immunized with free CPI
Sham immunized

FIGURE 2

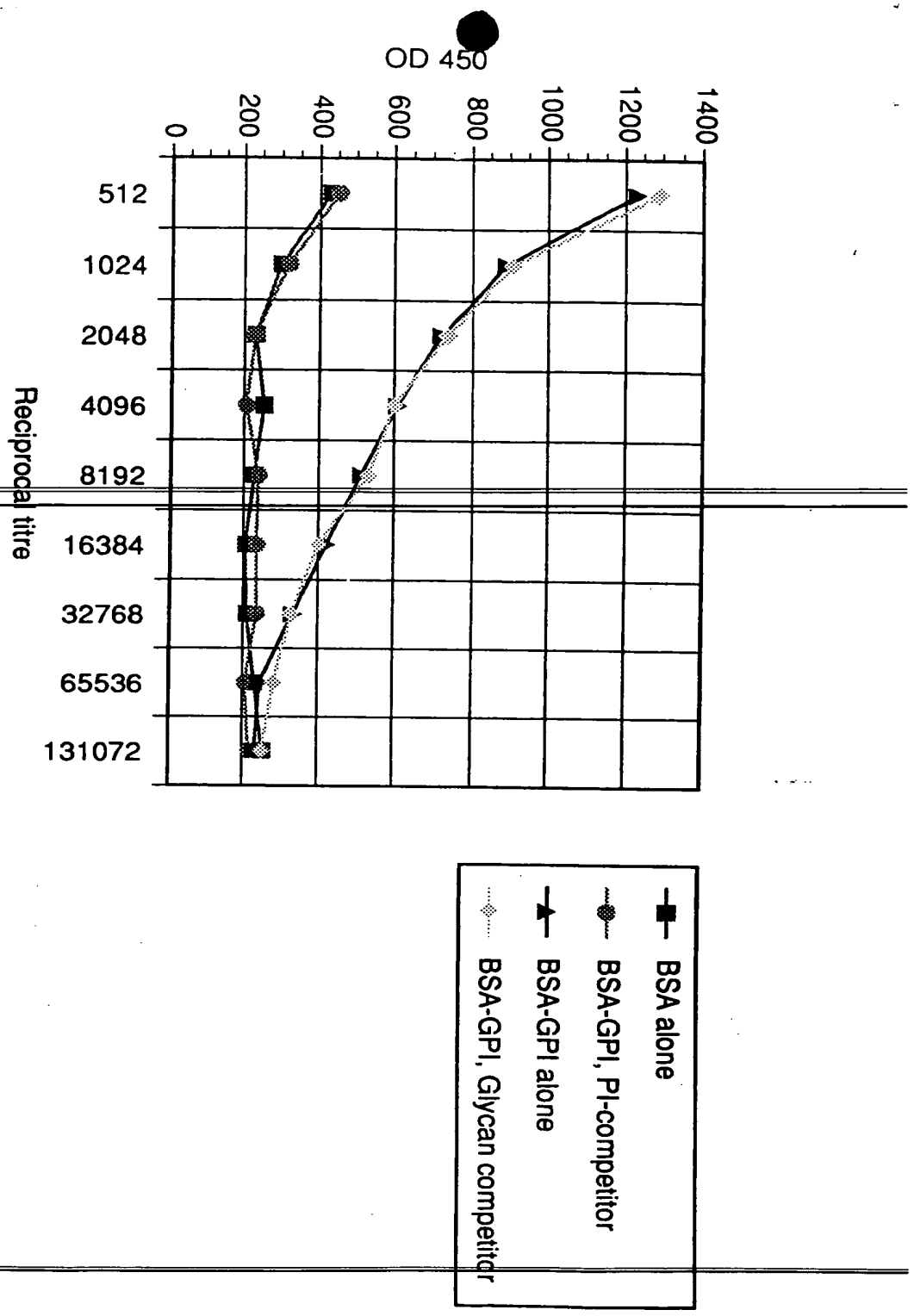
OD 450
0
200
400
600
800
1000
1200
1400
Reciprocal titre
512
1024
2048
4096
8192
16384
32768
65536
131072
BSA alone
BSA-GPI, PI-competitor
BSA-GPI alone
BSA-GPI, Glycan competitor

FIGURE 3

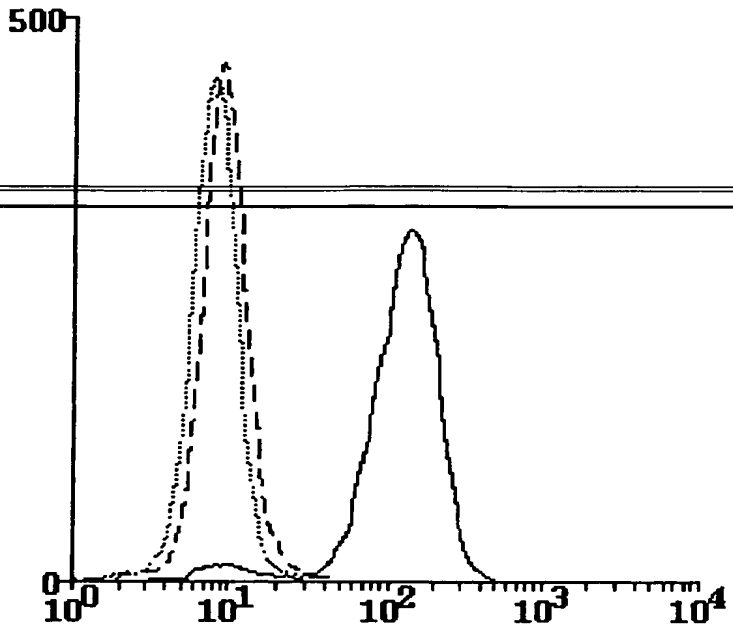

FIGURE 4

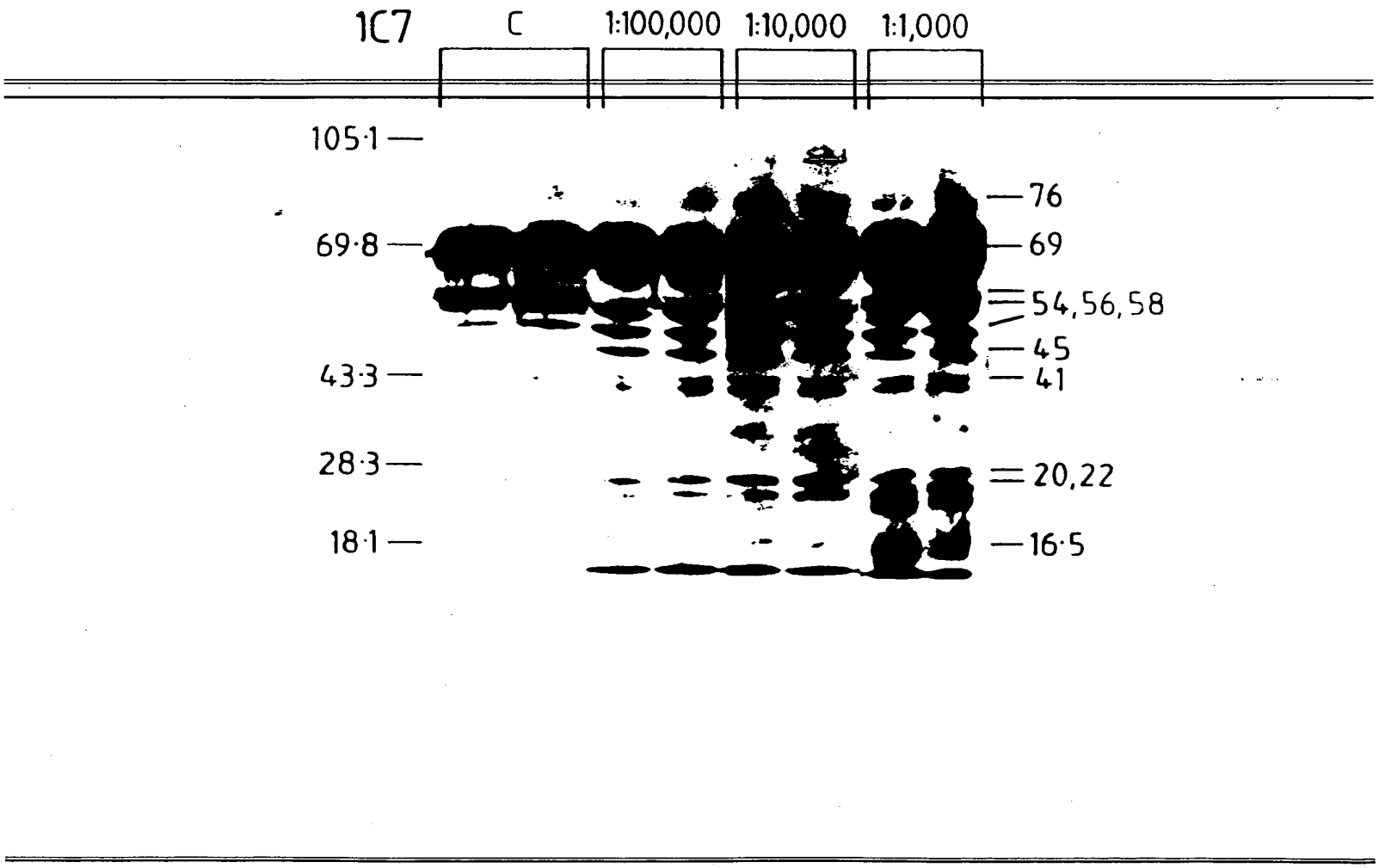
1C7
C
1:100,000
1:10,000
1:1,000
105·1
69·8
43·3
28·3
18·1
76
69
54,56,58
45
41
20,22
16·5

FIGURE 5

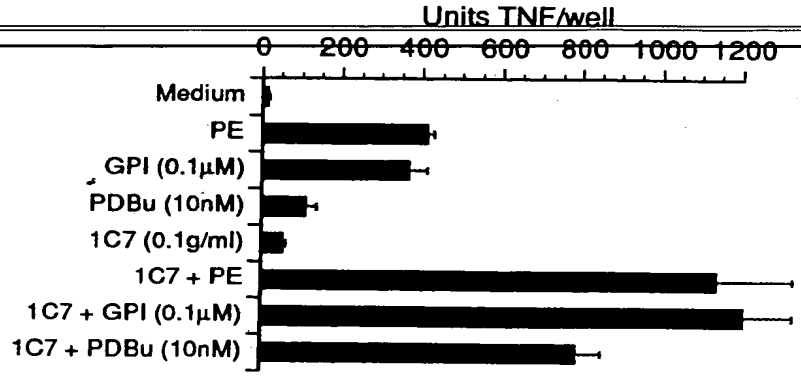
Units TNF/well
0
200
400
600
800
1000
1200
Medium
PE
GPI (0.1μM)
PDBu (10nM)
1C7 (0.1g/ml)
1C7 + PE
1C7 + GPI (0.1μM)
1C7 + PDBu (10nM)

FIGURE 6

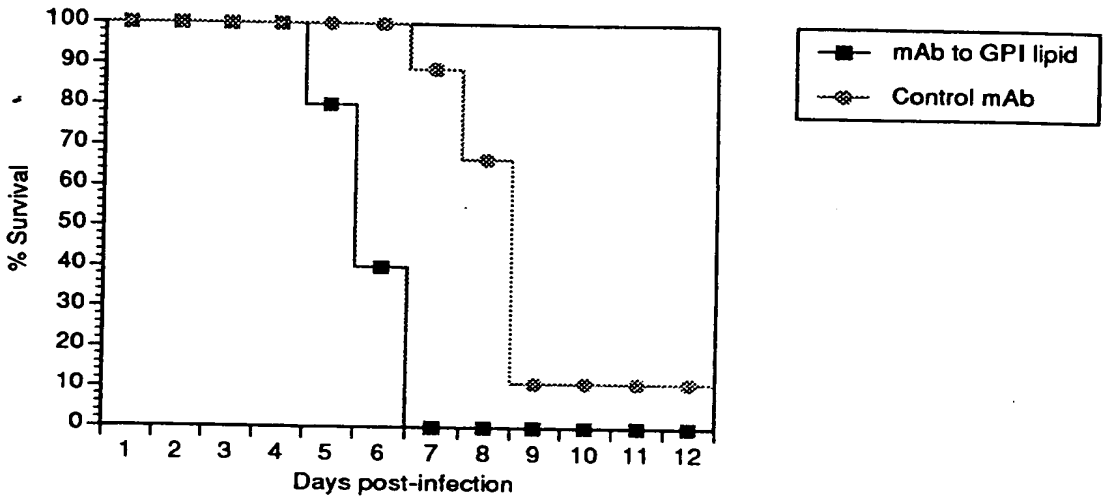
100
90
80
70
60
50
40
30
20
10
0
% Survival
1
2
3
4
5
6
7
8
9
10
11
12
Days post-infection
mAb to GPI lipid
Control mAb

FIGURE 7

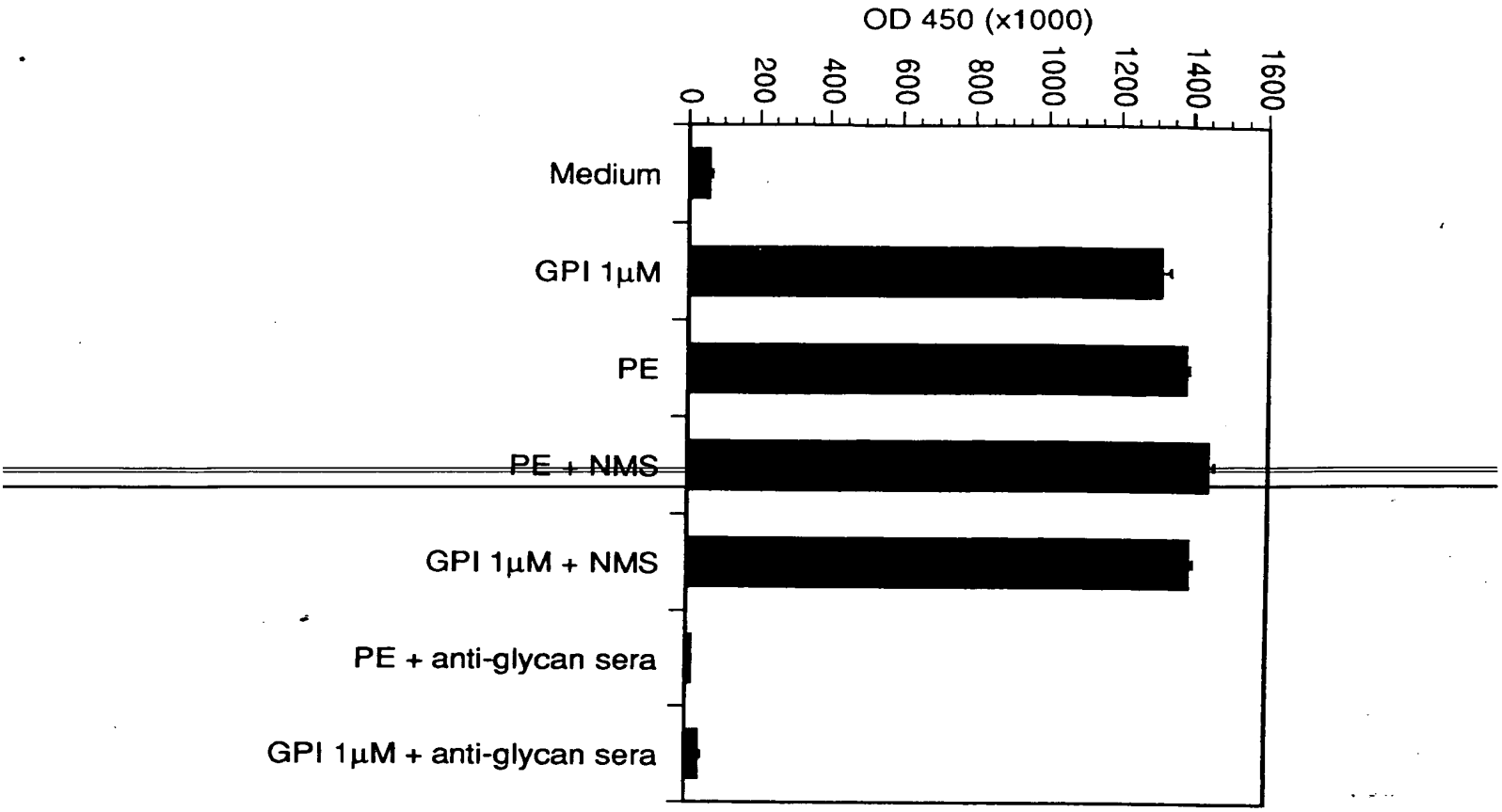
OD 450 (x1000)
0
200
400
600
800
1000
1200
1400
1600
Medium
GPI 1μM
PE
PE + NMS
GPI 1μM + NMS
PE + anti-glycan sera
GPI 1μM + anti-glycan sera

FIGURE 8

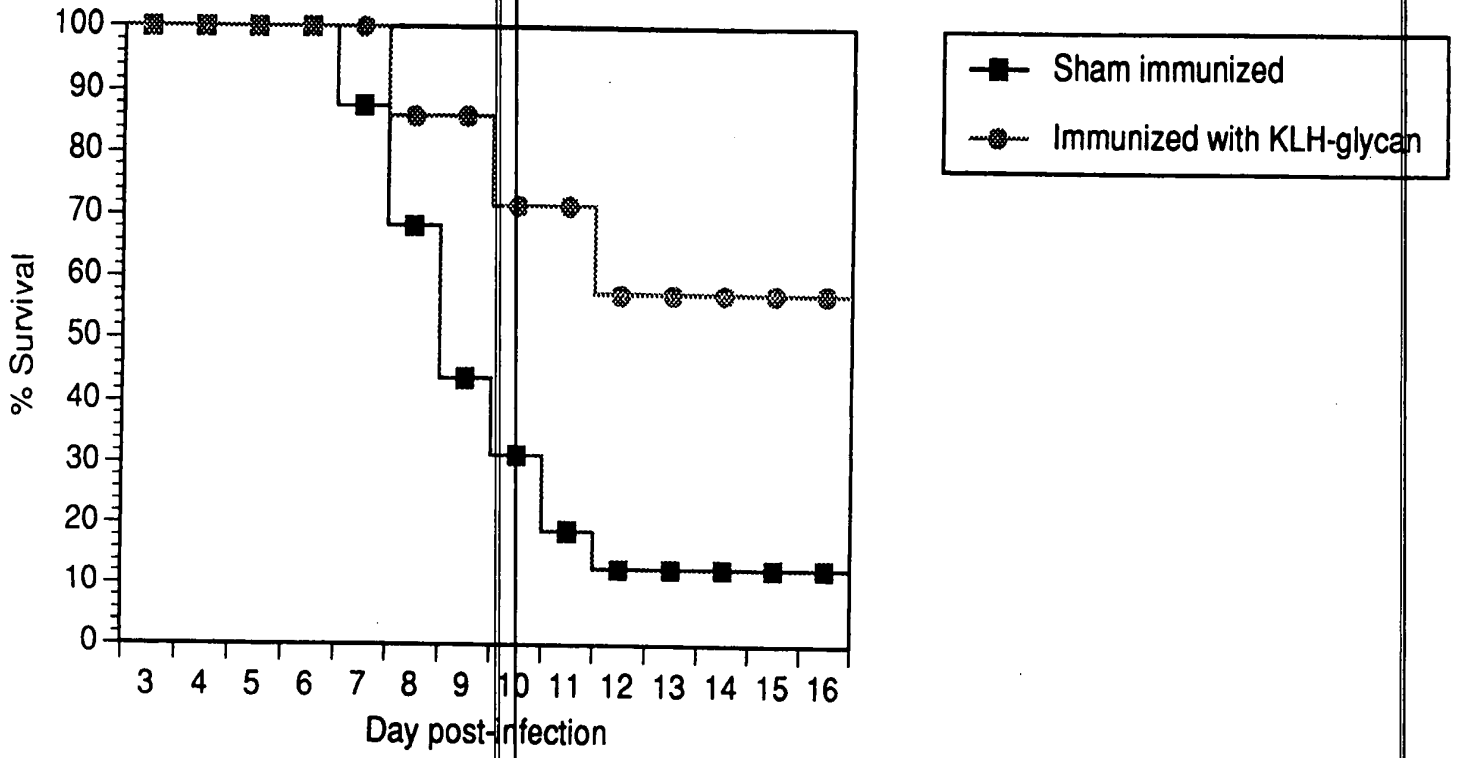
100
90
80
70
60
50
40
30
20
10
0
% Survival
3 4 5 6 7 8 9 10 11 12 13 14 15 16
Day post-infection
Sham immunized
Immunized with KLH-glycan

FIGURE 9

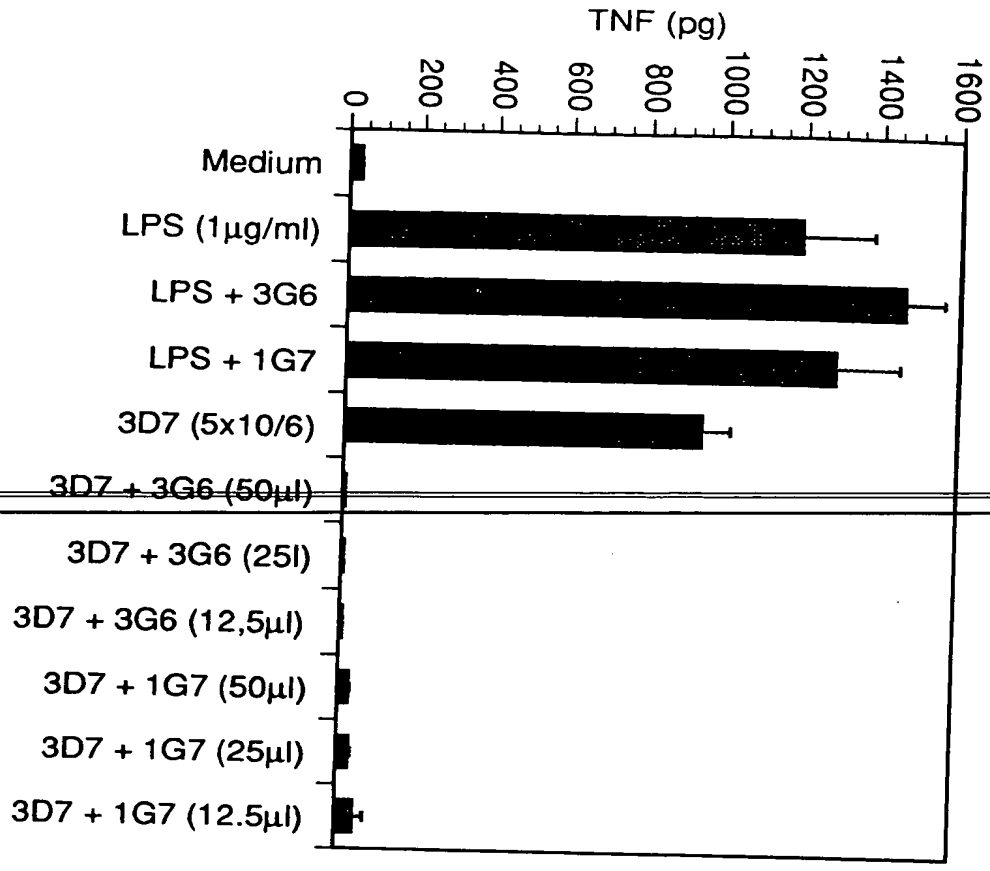
TNF (pg)
0
200
400
600
800
1000
1200
1400
1600
Medium
LPS (1µg/ml)
LPS + 3G6
LPS + 1G7
3D7 (5x10/6)
3D7 + 3G6 (50µl)
3D7 + 3G6 (25l)
3D7 + 3G6 (12,5µl)
3D7 + 1G7 (50µl)
3D7 + 1G7 (25µl)
3D7 + 1G7 (12.5µl)

FIGURE 10

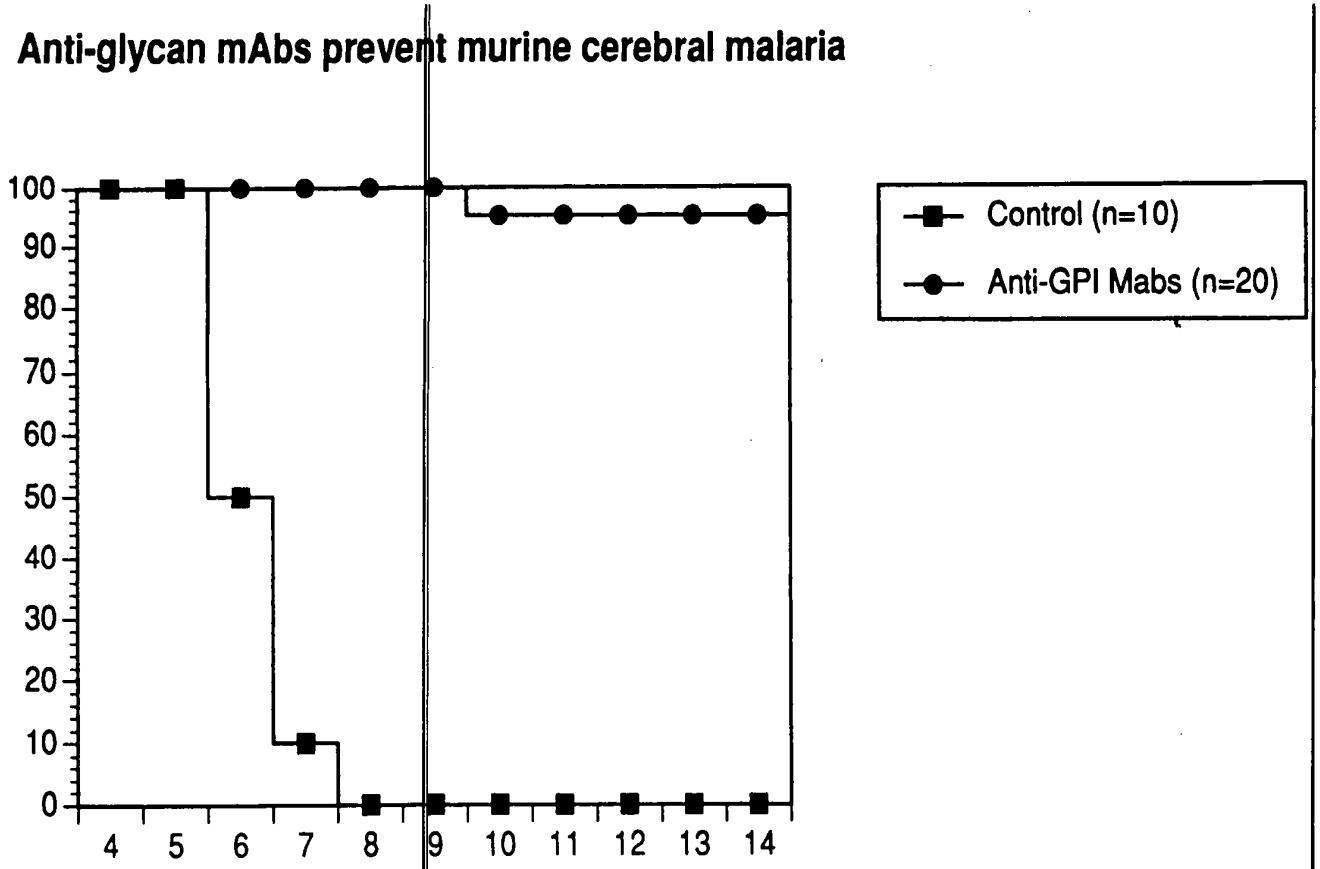
Anti-glycan mAbs prevent murine cerebral malaria
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
11
12
13
14
Control (n=10)
Anti-GPI Mabs (n=20)

FIGURE 11

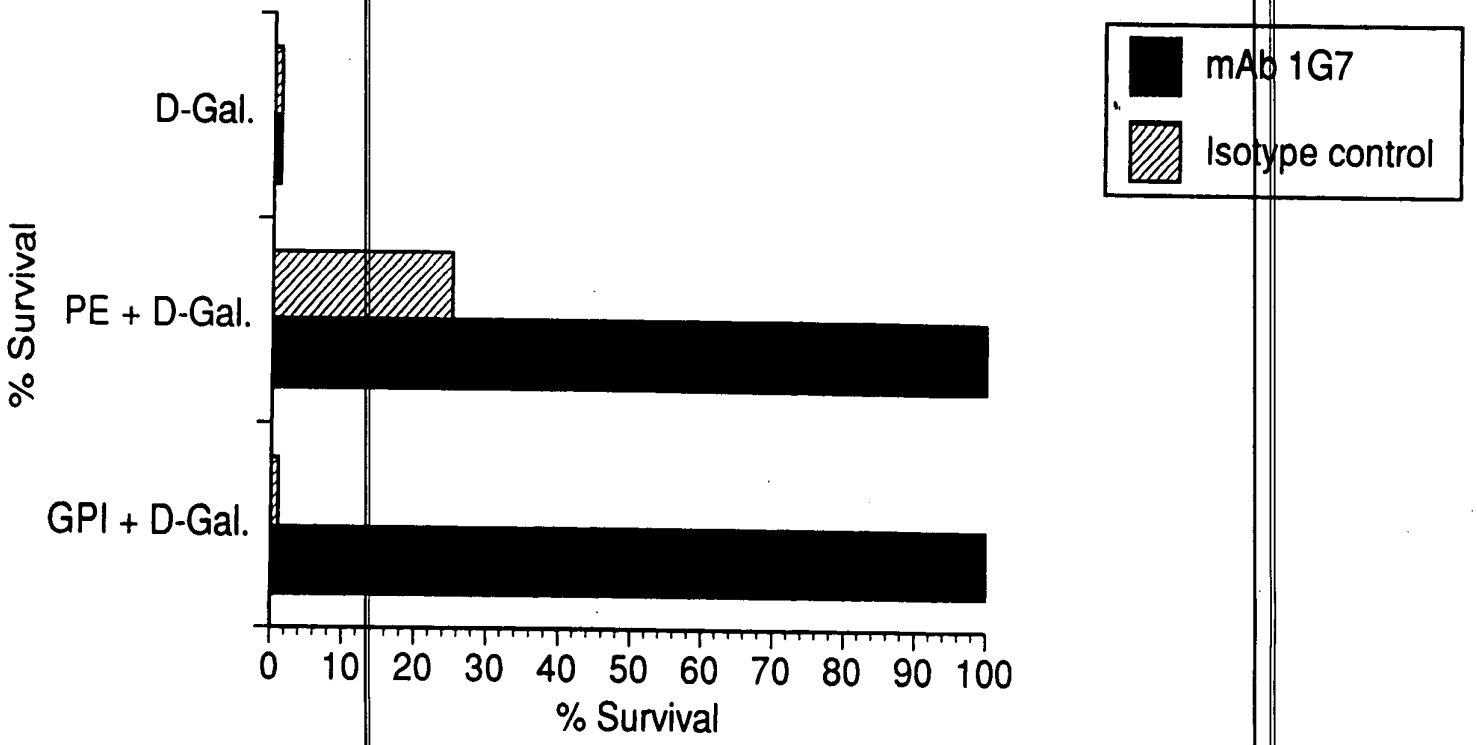
mAb 1G7
Isotype control
D-Gal.
PE + D-Gal.
GPI + D-Gal.
% Survival
0 10 20 30 40 50 60 70 80 90 100
% Survival

FIGURE 12